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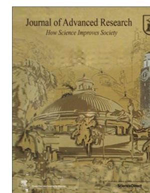

Original article

Preclinical studies of toxicity and safety of the AS-48 bacteriocin

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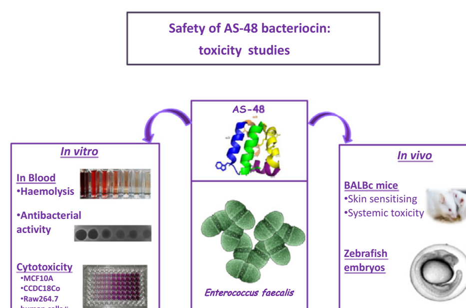
HIGHLIGHTS

- AS-48 is a 70-residue circular bacteriocin produced by *Enterococcus* strains.
- Toxicity of AS-48 in *in vitro* and *in vivo* models has been evaluated.
- The haemolytic activity and lacks of pro-inflammatory effects of AS-48 are minimal.
- AS-48 has scarce ability to cause loss of cell viability at therapeutic concentrations.
- AS-48 is a promising therapeutic agent against a vast array of microbial infections.

GRAPHICAL ABSTRACT



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ABSTRACT

The *in vitro* antimicrobial potency of the bacteriocin AS-48 is well documented, but its clinical application requires investigation, as its toxicity could be different in *in vitro* (haemolytic and antibacterial activity in blood and cytotoxicity towards normal human cell lines) and *in vivo* (e.g. mice and zebrafish embryos) models. Overall, the results obtained are promising. They reveal the negligible propensity of AS-48 to cause cell death or impede cell growth at therapeutic concentrations (up to 27 μM) and support the suitability of this peptide as a potential therapeutic agent against several microbial infections, due to its selectivity and potency at low concentrations (in the range of 0.3–8.9 μM). In addition, AS-48 exhibits low haemolytic activity in whole blood and does not induce nitrite accumulation in non-stimulated RAW macrophages, indicating a lack of pro-inflammatory effects. The unexpected heightened sensitivity of zebrafish embryos to AS-48 could be due to the low differentiation state of these cells. The low cytotoxicity of AS-48, the absence of lymphocyte proliferation *in vivo* after skin sensitization in mice, and the lack of toxicity in a murine model support the consideration of the broad spectrum antimicrobial peptide

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AS-48 as a promising therapeutic agent for the control of a vast array of microbial infections, in particular, those involved in skin and soft tissue diseases.
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Introduction

Among the diverse array of bacterial metabolites, the superfamily of antimicrobial ribosomal synthesised peptides (bacteriocins) is biotechnologically relevant for use as natural preservatives to extend the shelf-life of foods. They display remarkable broad-spectrum activity against Gram-positive and Gram-negative bacteria, including many antibiotic-resistant strains, often with EC_{50} values in the nano- to micromolar range. Currently, interest in peptides is increasing in pharmaceutical research and development. Approximately 140 therapeutic peptides are currently being evaluated in clinical trials [1]. Additionally, in contrast to many conventional antibiotics, most of them rapidly kill bacteria instead of just inhibiting bacterial growth. In general, this effect arises from their primary structure and properties such as positive net charge, amphipathicity, conformational flexibility, size, and hydrophobicity [2]. Although more than 335 bacteriocins have been described according to the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) [3], toxicity data has only been determined for a few. However, available research and their long track record of intentional use in food strongly suggest that these peptides can be safely used. Bacteriocins from lactic acid bacteria (LAB) exhibit low toxicity against most eukaryotic cells, which encourages their re-evaluation for clinical/veterinary use as antimicrobial drugs [4,5]. Their therapeutic potential in local and systemic bacterial infections is currently under investigation, underlining their importance as a viable alternative or addition to currently used antibiotics, considering the increasing spread of antibiotic resistance. Many bacteriocins fulfil most criteria, including low MIC values, low immunogenicity and toxicity, and low potential to induce resistance, which encourage studies aimed at marketing them as clinically relevant compounds [6–9]. Interestingly, some recent reports describe very potent bacteriocins produced by enterococci, such as enterocins A and B, produced by *Enterococcus faecium* [10], enterocins DD14 [11] and SL-5 [12], both identical to MR10A and MR10B [13], enterocin EntV [14] and even enterocin EF478 [15], which displays sequence similarity to a serine protease of *Enterococcus faecalis*. In no case has a thorough characterization of its therapeutic profile been conducted. Some studies of immunogenicity and *in vivo* toxicity have been reported for some lanthipeptides [16], peptide P34 from *Bacillus* [17], bacteriocins LR14 from *Lactobacillus plantarum* [18], and TSU4 from *Lactobacillus animalis* [19].

One of the most interesting classes of bacteriocins, based on physicochemical characteristics, is the head-to-tail circular peptides produced by Gram-positive bacteria [20]. These biologically active peptides are post-translationally modified by linkage of their N- and C-termini via peptide bond, yielding circular active molecules. They represent a group of antimicrobial peptides that exhibit broad antimicrobial activity and greater stability and resistance to exoproteases than their unmodified linear counterparts [20]. For these reasons and because they closely mimic natural pathways, they have remarkable therapeutic potential in local and systemic infections [21]. Another advantage is that their mechanism of action, which specifically targets the bacterial cell membrane, is novel compared to antibiotics in clinical use, and bacteria seem less capable of developing resistance towards these molecules [4,5]. The prototype circular bacteriocin is AS-48, a 70-residue α-helical peptide produced by different strains of *Enterococcus*, whose structure, biological activity, and genetic regulation, have been extensively examined [22]. This peptide exhibits a strong cidal/lytic activity against most pathogenic Gram-positive bacteria, including *Listeria monocytogenes* [23], *Staphylococcus aureus* [24], *Streptococcus agalactie* (unpublished data), *Mycobacterium tuberculosis* [25], and *Propionibacterium acnes* [26].

The objective of the present study was to characterise the toxicology of the circular bacteriocin AS-48, to determine if it is safe for clinical/veterinary use. The potency it exhibited against *S. aureus* and *P. acnes* clearly indicates the potential for the use of AS-48 in treating skin and soft tissue infections caused by these microorganisms. Thus, several biosafety parameters and its functionality have been analysed using different experimental *in vitro* models (blood, human, and mouse cell lines) and *in vivo* using murine and zebrafish embryo models. The novelty of this study lies mainly in the scarcity and limited scope of toxicological analyses of bacteriocins to date, with the exception of analyses of haemolysis and cytotoxicity towards tumour cell lines. Thus, to our knowledge, this is a pioneering article in the field of bacteriocin toxicity in *in vivo* models.

Material and methods

Bacteriocin AS-48 preparation

AS-48 was purified from supernatants of *Enterococcus faecalis* strain UGRA10 [26] cultured in Esprion 300 plus 1% glucose (E-300-G, DMV Int., Veghel, the Netherlands), using previously established conditions [27]. The bacteriocin was purified to homogeneity (up to 95% purity) by cation exchange and reversed-phase high-performance liquid chromatography (RP-HPLC) as described elsewhere [27]. The concentration of purified samples was measured spectrophotometrically at 280 nm using a Nanodrop 2000 (Thermo Fisher Scientific. Waltham, MA, USA), with the molecular extinction coefficient 12 490 M^{-1} cm^{-1} according to Gill and von Hippel [28], the measured molecular weight 7149.5 Da and the E&MW option.

Haemolytic assays

The haemolytic potential of AS-48 was spectrophotometrically measured by quantifying haemoglobin released, using whole blood and defibrinated erythrocytes from 20 human samples (10 males and 10 females) obtained from the Biobank of Granada (Spain) (http://www.juntadeandalucia.es/salud/biobanco/) (Public Sanitary System of Andalusia) after obtaining the appropriate permits from the Ethical Research Committees of the Biobank and the University of Granada (Application number 32130034PV01). Biological samples were used in compliance with current legislation. Blood samples from healthy human volunteers were provided in BD Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, Nueva Jersey, USA) with K_2-EDTA as anticoagulant.

For the haemolytic test in whole blood, AS-48 was directly added to yield different final concentrations (0.5, 1, 2.5, 5, 10, 15, and 20 μM) in 1 mL of fresh blood. Tubes were slowly stirred at 37 °C for 15 min, and cells were removed by centrifugation at 3000 rpm at 4 °C for 15 min. Supernatants were diluted 10 fold, and absorbance (OD) at 540 nm was measured, using fresh blood diluted 10 fold in MilliQ water as a positive lysis control.

To assay erythrolytic activity the protocol described by Huang *et al.* [29] was performed with some modifications. Briefly, 20 samples of human red blood cells were isolated from 5 mL of fresh whole blood by centrifugation at 3000 rpm. Pellets were rinsed twice in saline solution (0.9% NaCl). The final pellet was resuspended in 5 mL of saline solution. Erythrocytes were diluted 10 fold and 800 μL of this suspension was mixed with 200 μL of different dilutions of AS-48 samples to achieve final concentration of 0.5, 1, 2.5, 5, 10, 15, and 20 μM. Mixtures were maintained at 37 °C for 15 min with slow stirring. Haemoglobin release was quantitated by measuring optical density at 540 nm (OD_{540}) after centrifugation (6 min at 3000 rpm, 4 °C). Controls with 0 or 100% haemolysis were obtained by adding 200 μL of saline solution or MilliQ water, respectively. The percentage of haemolysis (% H) was calculated as follows: $\% H = 100 \times (A - A_0)/A_{tot}$, with A being the absorbance of the sample with added AS-48; A_0, the absorbance of the negative control (0.9% NaCl); and A_{tot}, the absorbance of the positive control (MilliQ water).

Antibacterial activity in blood

Fresh blood samples (0.8 mL) were pre-inoculated with 10^6 CFU/mL of susceptible Gram-positive bacteria species with well-known MICs (*Listeria monocytogenes* CECT4032, *Enterococcus faecalis* V583, *Staphylococcus aureus* CECT240 or *Streptococcus agalactiae* Hana-1), and the Gram-negative species *Klebsiella pneumoniae* UGRA-1 (data not shown). These bacterial strains were grown in brain heart infusion (BHI) or Luria broth (LB) prior to blood inoculation. The inoculated blood samples were added to different concentrations of AS-48 (0.014, 0.07, 0.14, 0.7 or 1.4 μM in accordance with their known susceptibilities). Cultures were incubated for 24 h at 37 °C with gentle shaking, and samples were taken at different times (0, 2, 4, 6, and 24 h). Residual CFU/mL of each indicator bacteria at each time point was determined to quantify death kinetics by plating them on appropriate solid media. All experiments were performed in triplicate.

Zebrafish embryos acute toxicity assay

The present test for short-term toxicity follows the guidelines of the OECD Draft Guideline Fish Embryo Toxicity (FET) Test for the study of chemical substances (May 30, 2006) [30]. The species chosen for this test was the zebrafish (*Danio rerio*) wild strain AB. Breeding fish were maintained according to specifications defined in the technical instruction IT-DD-17, and production of fish eggs for assays was performed according to the protocol PT-DD12 (Neuron Biopharma, Granada, Spain).

For this assay, normal fertilised eggs were deposited in a 24-well microplate (10 embryos per concentration). Five final concentrations of AS-48 (0.6, 1.4, 3.0, 6.4, and 14.0 μM) were evaluated, using 3,4-dichloroaniline (3,4-DCA) as a positive control and water as a negative control. Plates were incubated at 26 ± 1 °C for 24 and 48 h post fertilization (hpf), then the embryos were checked for lethal, sublethal, or teratogenic effects in accordance with procedure outlined by Lammer *et al.* [31].

In vitro Cytotoxic effects against human cell lines

The effect of AS-48 on the viability *in vitro* of eukaryotic cells was assessed in the Cytotoxicity Service of the Medina Foundation (Granada, Spain) using the colorimetric MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay [32]. Reduction of tetrazolium to insoluble formazan by cellular enzymes generates a purple colour. Different concentrations of AS-48 (from 0.27 to 27 μM) were tested against the non-tumour epithelial cells MCF10A (ATCC® CRL-10317) and colonic fibroblast cells CCD18Co (ATCC® CRL-1459). Cells were inoculated into 96-well plates (1×10^4 cells/well in 200 μL of appropriate culture media) and incubated overnight at 37 °C in 5% CO_2. Then, the medium was replaced, and cells were treated with different concentrations of AS-48 for 24 h. As positive and negative controls, 8 mM methyl methane-sulfonate (MMS) and 0.5% DMSO were used, respectively, with doxorubicin as an internal control. Upon treatment with controls or AS-48, plates were incubated at 37 °C in 5% CO_2 for 72 h. The media was then removed from the wells, and MTT was added, followed by a 3 h incubation period. Dimethyl sulfoxide (DMSO) was added to solubilise the formazan crystals, and the absorbance of the obtained solutions was measured at 570 nm using a multiReader Victor TM (Perkin Elmer Waltham, MA. USA). Viability data are depicted as the mean percentage ± standard deviations compared to the control (100%). The results are averages of three experiments.

Cell viability and nitrite production in treated macrophage RAW cells

The RAW264.7 mouse macrophage tumour cell line was purchased from the Cell Culture Unit of the University of Granada (Granada, Spain), and cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal bovine serum and 2 mM L-glutamine, in a humidified 5% CO_2 atmosphere at 37 °C. The effect of AS-48 on cell viability was measured using the Cell Titer 96 s AQueous One Solution Cell Proliferation Assay (MTS) from Promega (Madison, WI, USA). Briefly, cells were seeded in 96-well plates and incubated for 2 h with AS-48 (0.27–27 μM), they were then challenged with 100 ng/mL lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 for 24 h to induce expression and release of cytokines/chemokines. Then, the MTS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was added to the wells, and absorbance was measured at 490 nm after 1–4 h at 37 °C in a Dynex spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Cell viabilities were obtained by comparing measured absorbances with the absorbance of the control cells.

The effect of AS-48 on nitrite production was also evaluated using cells cultured as described above. After 2-h incubation with AS-48 followed by 24-h incubation with LPS, cell supernatants were collected, and nitrite levels were quantified by the addition of Griess reagent (a coloured azo dye product). Its absorbance was measured at 540 nm in a Dynex spectrophotometer (Dynex Technologies, Chantilly, VA, USA) [33].

Local lymph node assay: BrdU-ELISA

Experimental procedures and protocols used in this study were performed according to the "Guide to the care and use of laboratory animals in research and teaching" [34]. The Commission of Ethics in Animal Experimentation of the University of Granada (Spain) approved the experimental protocol (Register number 14-CEEA-OH-2013).

Twenty five CD1 female mice (18–22 g) purchased from the Laboratory Animal Services of the University of Granada and maintained in Makrolon cages were provided with food and water *ad libitum* in an air-conditioned atmosphere with a 12-h light-dark cycle. The mice were randomly allocated into five groups (five animals per group): three groups treated with three different doses of AS-48 (20, 10, or 1 μg/mouse), a non-treated control group, and a group treated with phorbol-12-myristate-13-acetate (PMA) as positive control. Five microliters of AS-48 in the appropriate DMSO solution was applied to the dorsum of both ears daily for 3 consecutive days, whereas control groups received only vehicle. On day 5, all mice received a single intraperitoneal injection (0.5 mL) of 5-bromo-2-deoxyuridine (BrdU) solution (8 mg/mL). On day 6, a

pair of auricular lymph nodes from each mouse was excised to measure incorporation of BrdU into lymph node cells using a CytoSelect™ BrdU Cell Proliferation ELISA Kit from Cell Biolabs Inc. (San Diego, CA, USA). Briefly, lymph nodes were minced and resuspended in 20 mL of PBS solution that had been filtered through a 100-μm mesh. Aliquots of 100 μL were added to a 96-well flat bottom plate, which was centrifuged at 300*g* for 10 min. Seventy five microlitres was removed from each well, and the plate was dried at 60 °C for 1 h. Then, anti-BrdU antibody was added, and the protocol provided by the kit manufacturer was followed. BrdU content in each well was measured by reading the absorbance at 450 nm in a Dynex spectrophotometer (Dynex Technologies, Chantilly, VA, USA).

In vivo testing of AS-48 in BALB/c mice

Assays were performed according to rules and principles of the international guide for biomedical research in experimental animals, including use of the minimum number of animals necessary to produce statistically reproducible results, and the Three Rs concept (Replacement, Reduction, and Refinement). Seven female BALB/c mice (10–12 weeks old and ~21 g) were selected for these experiments; they were maintained under standard conditions and provided with water and standard chow *ad libitum*. Four mice were treated with a total of 5 mg/kg (100 μg/mouse) of AS-48 divided into six doses and administered every 8 h. Mice were intraperitoneally injected 200 μL of 11.65 μM (83.34 μg/mL) AS-48 dissolved in water. Three negative control mice were administered only vehicle. After the final injection, the weight of the mice was determined every 2 days.

Mouse serum samples were obtained 48 h and 7 days after AS-48 treatment as described Martín-Escolano *et al.* [35]. Kidney, heart, and liver marker profiles in serum samples were measured using a commercial kit from Cromakit® with a BS-200 Chemistry Analyzer (Shenzhen Mindray Bio-medical Electronics Co., Ltd, Shenzhen, China) in the Scientific Instrumentation Service at the University of Granada. Finally, two AS-48-treated mice were euthanised 48 h after treatment, and their spleens were harvested and weighed to evaluate inflammation. Seven days after treatment, the same procedure was carried out for the other two AS-48-treated mice and the negative control mice.

Statistical analysis

Statistical analyses were performed using IBM SPSS version 20 (IBM, Spain). Three independent tests were conducted for each experiment, and data are expressed as mean ± standard deviation (SD). SPSS one-way ANOVA and LSD post-hoc test were performed for statistical data analysis. The *t*-test for paired samples was used to assess whether there were differences between the assays used, with $P < 0.05$ considered statistically significant at a 95% confidence level. Statistical studies based on contingency tables (prevalence) together with the ×2 test for relationships between variables were also conducted.

Results

Haemolytic activity

The haemolytic potential of purified AS-48 was determined by the percentage of lysed red cells using whole blood and defibrinated human erythrocytes. According to the data obtained, the haemolytic potential of purified AS-48 varied with assay and concentration used. When tested on whole blood, hemolysis produced by the highest concentrations used (20 μM) was very low (less than 1.2%). Practically zero haemolysis occurred at concentrations of 5 μM or lower (Fig. 1).

With defibrinated erythrocytes, the percent haemolysis was 3% at AS-48 values close to 5 μM, which increased to 15.7 and 22.6% at 10 and 20 μM, respectively (Fig. 1). It is remarkable that lysis caused by AS-48 on defibrinated erythrocytes required concentrations much higher than those required for antimicrobial activity.

Bactericidal activity of AS-48 in human blood

To investigate the stability and bactericidal activity of AS-48 in whole human blood, four species of Gram-positive bacteria (*L. monocytogenes* CECT4032, *E. faecalis* V583, *S. agalactiae* Hana-1

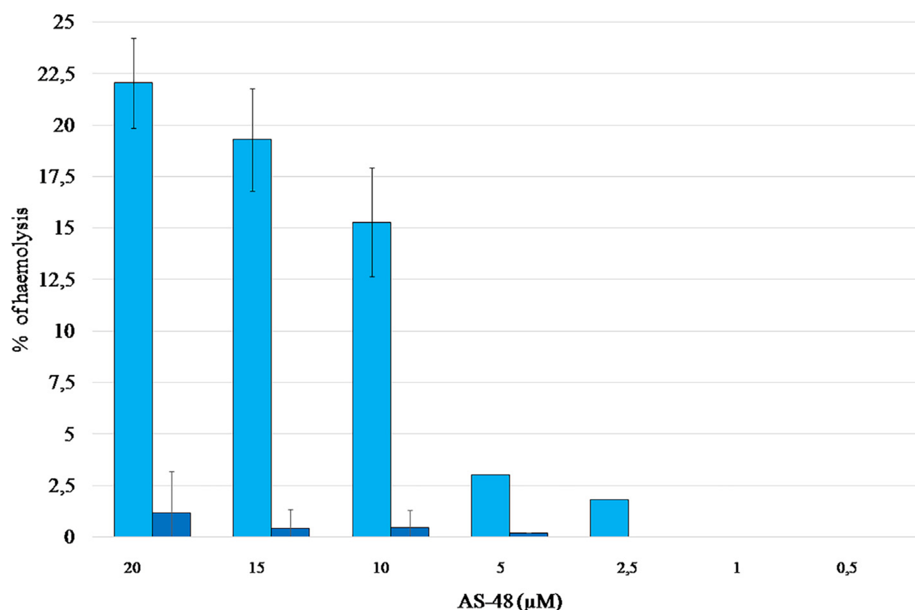

Fig. 1. Percentage of haemoglobin released on exposure to different AS-48 concentrations using whole blood (dark blue) and defibrinated erythrocytes (light blue). The results are shown as means ± SD of twenty independent samples.

and *S. aureus* CECT240) and one Gram-negative species (*Klebsiella pneumoniae* UGRA-1) were selected, and their CFU/mL in blood was monitored over the course of 24-h exposure to different concentrations of AS-48. AS-48 concentrations were chosen based on previously determined MIC values for each bacterium (data not shown).

A remarkable dose-dependent reduction in bacterial counts (Fig. 2), which varied with the indicator bacteria used and the AS-48 concentration added, was observed. Thus, 0.14 μM of AS-48 completely eradicated *L. monocytogenes* after 6 h, whereas *S. aureus* CECT240 required 24-h incubation. At lower concentrations (0.014 and 0.07 μM) CFU/mL decreased in the first 6 h of incubation, but total elimination of these bacteria was not observed (in both cases, approximately 1×10^4 CFU/mL remained after 24 h). To eradicate *E. faecalis* V583 and *S. agalactiae* Hana-1, 10-fold higher concentrations of AS-48 (1.4 μM) were required to achieve the same effects seen with *L. monocytogenes* and *S. aureus* (i.e. eradication after 6 and 24 h, respectively). These results demonstrate that AS-48 is stable in blood for at least 24 h and that its bactericidal potential remains intact in the presence of blood. As expected, no inhibition of growth was observed at any AS-48 concentrations assayed with *K. pneumoniae* UGRA-1, which is resistant to AS-48 (Fig. 2) [36].

Assessment of AS-48 toxicity in zebrafish eggs

Zebrafish embryos, which are very sensitive to toxins, were used to assess the safety, bioavailability, and efficacy of AS-48. Embryos exposed to low doses (0.6–3.0 μM) did not display any visible anomalies after 24 h (Fig. 3).

At concentrations <3 μM, AS-48 was bio-safe even after 48 h of treatment. At 3 μM, oedema was produced after 48 h in 33.3% of embryos (Table 1 and Fig. 3). The lethal dose 50 (LD_{50}) was established to lie between 3.0 and 6.4 μM, and the maximum tolerated dose (MTD), at which no toxic or lethal effect could be detected, was 1.4 μM (10 μg/mL). It is noteworthy that higher doses (6.4 and 14 μM) induced 100% embryo lethality at 24 hpf, during the most critical stage of embryonic development, when organogenesis takes place [37], with filamentous structures visible on the surface of the embryos' chorion. This lethality could be due to a membrane dysfunction, calcium imbalance, or outward blebbing of the cell membrane [38].

In vitro toxicity and pro-inflammatory effects of AS-48

The *in vitro* cytotoxicity of the antimicrobial peptide AS-48 at concentrations ranging from 0.27 to 27 μM was investigated on two primary human cell lines (CCD18Co and MCF10A) using the MTT assay, which measures the mitochondria-dependent metabolic activity of cells. At concentrations close to the MIC, the presence of AS-48 did not affect these non-tumour human-skin cell lines. No signs of viability changes were observed even at the highest concentration tested (27 μM) (Fig. 4). Statistical analysis showed no significant differences between the treated cells and the negative control, confirming the lack of AS-48 toxicity toward these cell lines under the tested assay conditions (Fig. 4).

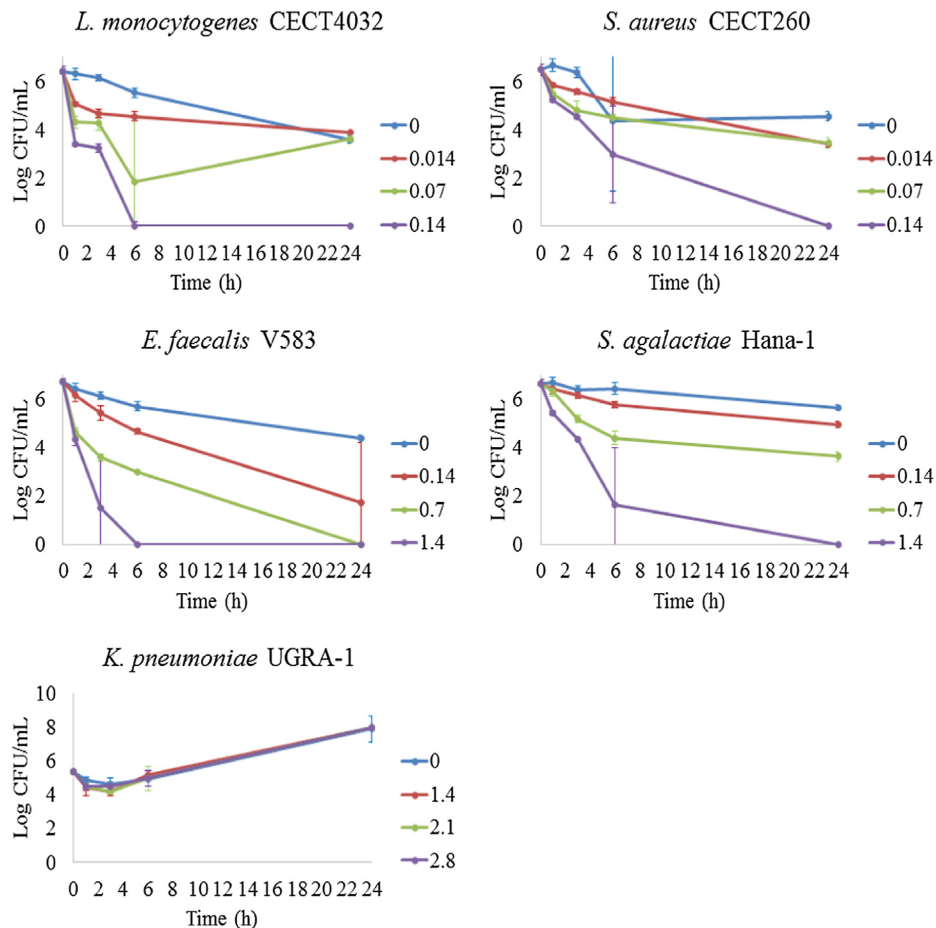

Fig. 2. Bactericidal activity of AS-48 against susceptible (*Listeria monocytogenes* CECT4032, *Enterococcus faecalis* V583, *Streptococcus agalactiae* Hana-1 and *Staphylococcus aureus* CECT240) and resistant bacteria (*Klebsiella pneumoniae* UGRA-1) previously inoculated (10^6 CFU/mL) in human blood. Growth in blood in the presence of different concentrations of AS-48 was monitored during 24 h.

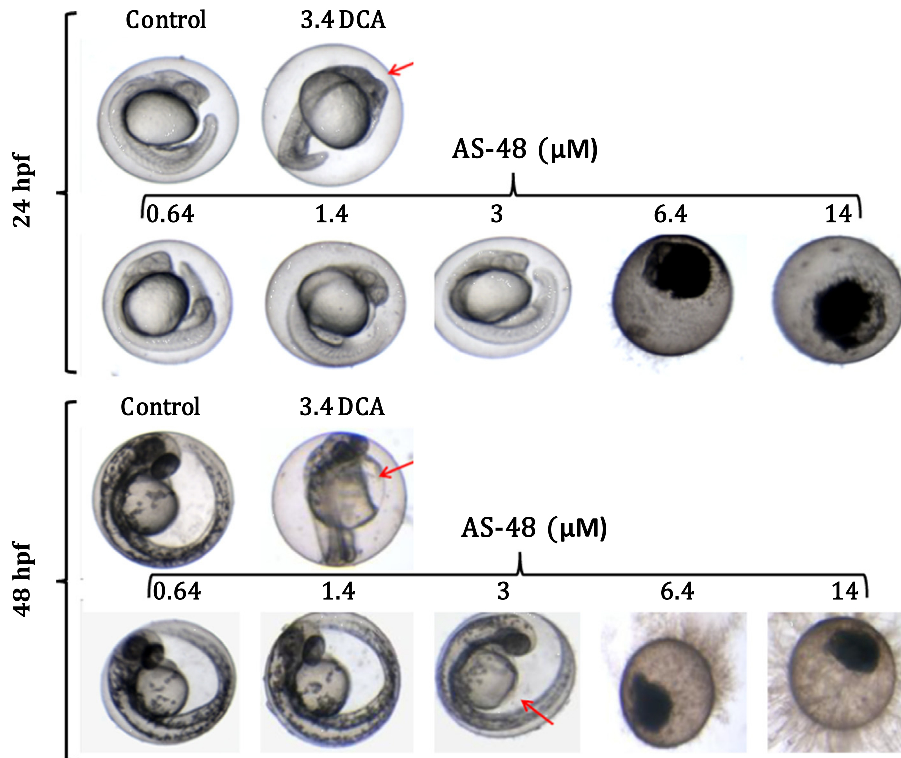

Fig. 3. Representative stereomicroscopic images of zebrafish embryos exposed to different AS-48 concentrations for 24 and 48 hpf. Images of the negative control (in water) and the positive control with dichloroaniline (DCA) are also displayed. The images were acquired at 32×. Red arrows indicate the formation of oedema that was observed in 33.3% of the treated embryos. The highest AS-48 concentrations tested (6.4 and 14 μM) induced a lethal effect with filamentous structures on the surface of the embryos chorion.

Table 1
Percentage of embryos that displayed some type of effect according to AS-48 concentration and time employed (24 and 48 hpf), using 3,4-dichloroaniline (3,4-DCA) as a positive control and water as a negative control.

AS-48	Cumulative mortality (%)		Sub-lethal effects (%)		Teratogenic effects (%)	
	24 hpf	48 hpf	24 hpf	48 hpf	24 hpf	48 hpf
0.64 μM	0	0	0	0	0	0
1.39 μM	0	0	0	0	0	0
3 μM	0	30	0	33.3	0	28.6
6.43 μM	100	100	100	100	100	100
14 μM	100	100	100	100	100	100
Control –	0	0	0	0	0	0
Control +	0	60	100	100	100	100

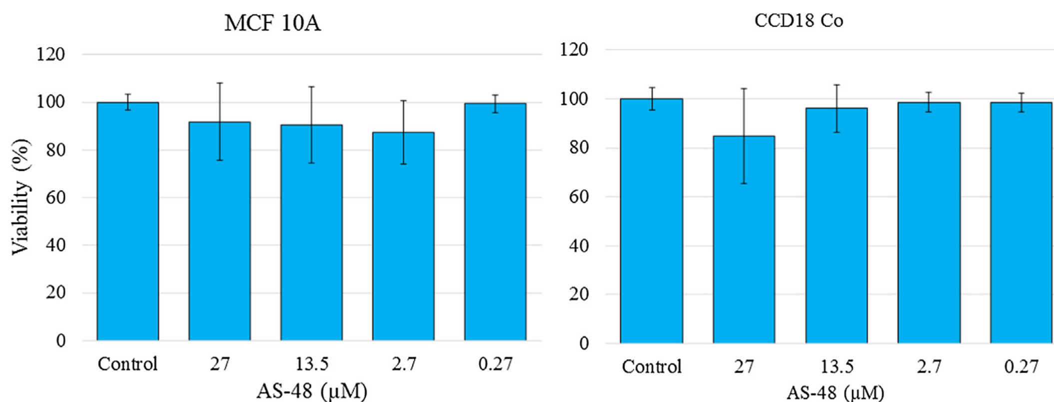

Fig. 4. Viability of MCF10A and CCDC18Co human cells lines (% in relation to control) after treatment with different concentrations of AS-48. C: control, untreated cells.

Similarly, RAW264.7 murine macrophages were exposed to increasing concentrations of AS-48 (0.27–27 μM) to assess its cytotoxicity and possible pro-inflammatory effects. The results showed that AS-48 had no cytotoxic effects on this tumour cell line at the concentrations assayed. No significant reduction in cell viability was observed in these experimental conditions (Fig. 5A).

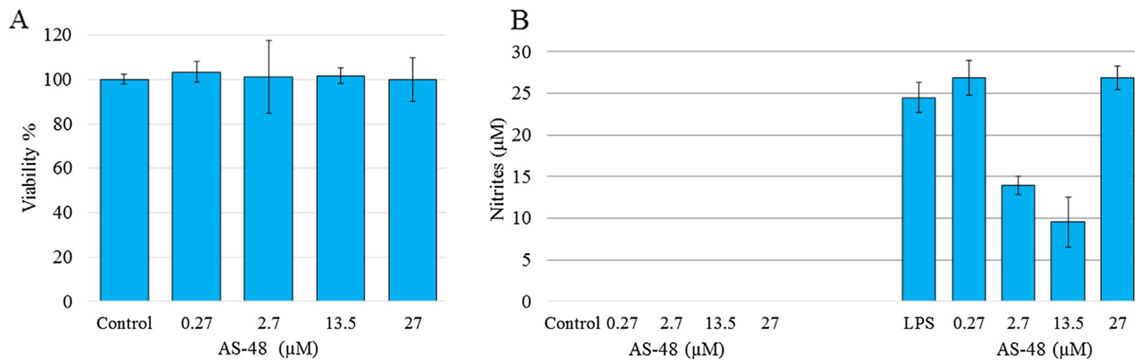

Fig. 5. AS-48 effect on the viability of the RAW264.7 cells (A) before and after LPS (100 ng/mL) stimulation (B). Data are expressed as means ± SD. The experiments were three times performed, with each individual treatment being run in triplicate.

Activated macrophages can overproduce nitric oxide (NO). This phenomenon has been associated with diverse inflammatory diseases ranging from tissue damage to sepsis [39]. Thus, the potential pro-inflammatory effect of AS-48 was evaluated by measuring nitrite accumulation in the cell culture medium (an indicator of nitric oxide synthesis by inducible nitric oxide synthase, also known as iNOS, after macrophage stimulation). In fact, nitric oxide production is up-regulated in dermal inflammatory reactions, and its role as a mediator of the irritant response is well documented [39]. Incubation of RAW264.7 cells with AS-48 (0.27–27 μM) did not induce nitrite accumulation. Nitrite levels in the media were similar in treated and control cells ($P > 0.05$) (Fig. 5B). However, when nitric oxide production was induced by LPS (100 ng/mL), AS-48 had a moderating effect, decreasing its production in a dose-dependent manner (Fig. 5B). The highest evaluated dose of AS-48 did not have any inhibitory effect, which could in principle seem paradoxical. However, this is a common phenomenon when dealing with natural products such as flavonoids. These show immunomodulatory properties, ameliorating inflammatory markers at low doses while showing no effect at higher ones [40].

Skin sensitizing potential of AS-48

Exposure to a dermal sensitiser results in T-cell proliferation in local lymph nodes, which can be measured *in vivo* by the incorporation of bromo-deoxyuridine (BrdU) [41]. This assay is widely used to identify products that may cause allergic contact dermatitis. In this work, the skin sensitizing potential of AS-48 was evaluated *in vivo* by applying different doses of AS-48 (1, 10, and 20 μg/doses) or vehicle (20 μL of DMSO) to the dorsum of both ears of CD-1 mice for 3 consecutive days, and then measuring lymphocyte proliferation in local lymph nodes by the BrdU assay and ELISA. The effect of BrdU incorporation on lymphocytes did not significantly differ in the AS-48 treated mice from that in the non-treated controls ($P > 0.05$), while PMA-challenged mice showed a significantly stronger signal ($P < 0.05$ *vs* untreated control) (Fig. 6).

In vivo AS-48 toxicity in mice: Tolerability and biochemical analysis

In vivo assays were performed to evaluate AS-48 toxicity at high concentrations (5 mg/kg) according to several serum biochemical measurements (% variation of uric acid, urea, creatine kinase-muscle/brain, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and bilirubin) as indicators of metabolic disturbances or abnormalities associated with treatment. AS-48 administration caused an alteration in biochemical parameters in comparison with the negative control mice, since tolerability trials are based on aggressive treatment

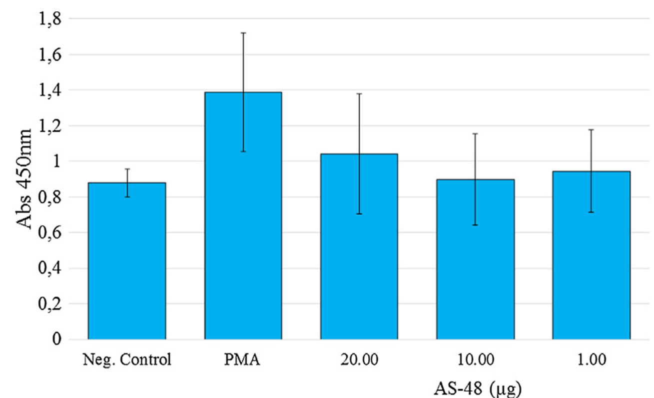

Fig. 6. Skin sensitizing potential of AS-48 evaluated *in vivo* by applying 1, 10, and 20 μg/doses of AS-48 (5 μL) to the dorsum of both ears of CD-1 mice for 3 consecutive days. The proliferation of local lymph node T cells was measured by incorporation of bromodeoxyuridine (BrdU) by reading the absorbance at 450 nm in the AS-48 treated mice. Negative (DMSO) and positive (PMA) treated controls.

in terms of dose and treatment regimen (Fig. 7). Most of these parameters returned to normal levels (<20% variation) within 7 days.

Remarkably, none of the treated mice died or lost more than 5% of their body mass, and all mice returned to their pre-treatment mass within 7 days. Spleens were weighed to evaluate splenomegaly as a possible response to AS-48, since this organ is involved in humoral and cellular immunity against infections. Weight percentages of spleens were determined for AS-48-treated (48 h and 7 days post-treatment) and negative control mice (7 days post-treatment) (Fig. 8). No significant differences were observed for the different groups of mice, demonstrating that splenomegaly did not occur in the treated mice.

Discussion

The emergence of bacterial resistance to antibiotics, a priority research area for the National Institute of Health (NIH) and the World Health Organization (WHO), necessitates the development of novel antimicrobial therapies: "*novel drug formulations with novel antimicrobials with unique mechanisms of action and new targets*". Although some promising agents are currently advancing, great difficulty is being faced in research on antibacterial drugs with regard to identifying new molecules with significant antimicrobial activity [42]. The reduced effort by pharmaceutical companies in the last 25 years to develop new antibiotics is adding to this growing crisis. However, renewed interest in discovery and development of new drugs has been observed in the last decade. Much

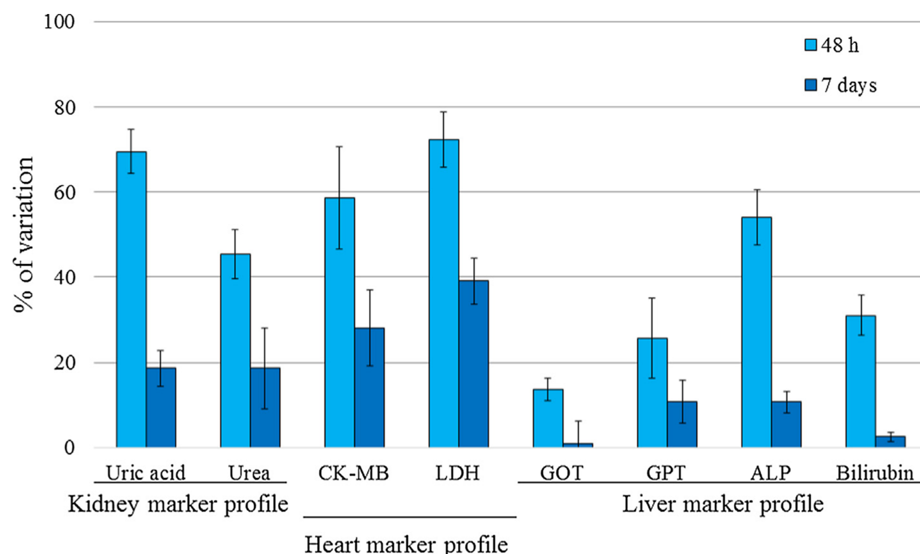

Fig. 7. Variation (%) of biochemical clinical parameters in mice, measured 48 h and 7 days after AS-48 treatment in comparison to a negative control. Data are expressed as means ± SD of four (48 h) and two (7 days) independent samples. CK-MB: creatine kinase-muscle/brain; LDH: lactate dehydrogenase; GOT: aspartate aminotransferase; GPT: alanine aminotransferase; ALP: alkaline phosphatase.

of this effort is focused on many kinds of natural products that are being extensively studied due to their easy accessibility, few side effects, low toxicity, and better biodegradability compared to other available antimicrobial agents, justifying the entry of some of these products into clinical trials [43].

Broad-spectrum antimicrobial peptides (AMPs), such as LAB bacteriocins, constitute a promising and exciting therapeutic option to treat MDR pathogens. Their specificity against microorganisms translates into excellent safety, tolerability, and efficacy profiles in humans in most cases [21]. The mechanism of action of most bacteriocins is mainly dependent on their amphiphilic nature and capacity to insert into and selectivity disrupt bacterial cytoplasmic membranes [2]. Several bacteriocins (e.g. salivaricin, nisin A, mersacidin, lacticin 3147, and leucocin A) have been tested against multi-drug resistant bacteria and even as immune modulators in treating hospital-acquired infections of the skin and mucosal wounds, since bacterial membranes have been revealed to be an important target, even for treating persistent infections [44]. Although efficacy in (mostly) *in vitro* and/or *in vivo* models has been tested for many bacteriocins, a thorough characterization of their fate upon administration is scarce. PK/PD studies reported

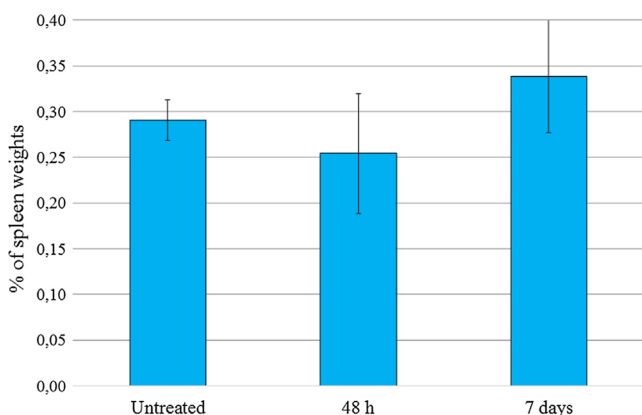

Fig. 8. Percentage of spleen weight for negative control mice (untreated) and AS-48-treated mice 48 h and 7 days after the treatment. Data are expressed as means ± SD of three (untreated), four (48 h) and two (7 days) independent samples.

end in most cases at the level of toxicity, without a careful assessment of administration, distribution, metabolism and excretion and their impact on efficacy.

One feature of many AMPs that causes much controversy and complicates drug development is that their antimicrobial activity is highly sensitive to environmental conditions. Other unfavourable properties include their low metabolic stability and their haemolytic activity (which are inherent risks of therapeutic peptides in general) together with salt sensitivity and high cost of production [45]. Collectively these are key factors limiting their clinical application. Remarkably, these features are not relevant for AS-48, whose cyclic structure confers surprising molecular flexibility, stability (even in the skin, where Na^+ is stored in large amounts), and resistance to exoprotease degradation, which is advantageous in therapeutic peptides. The broad antimicrobial spectrum of the bacteriocin AS-48 at low concentrations [22,25,26] promotes its application as a new drug for the treatment of infections caused by microorganisms, including antibiotic-resistant ones [4,24,26].

Owing to its promise as an antimicrobial agent, the behaviour of AS-48 has been studied in *in vitro* and *in vivo* models to evaluate if it exhibits some degree of selective toxicity (haemolytic activity, stability and antibacterial activity in blood, or toxicity against normal human cell lines, zebrafish embryos, and a murine model). The *in vitro* cytotoxicity assays are indicative of the intrinsic ability of a compound to cause cell death, defining concentration ranges for safe and rational administration. Overall, these results prove that AS-48 is safe against fresh human blood at concentrations >10-fold higher than those needed to inhibit the least sensitive species tested in this work (1.4 μM to eradicate *E. faecalis* and *S. agalactiae*). Thus, at 20 μM, red blood cell lysis was less than 1.2%, although with defibrinated erythrocytes, haemolytic activity increased (22.6% at the same concentration). Nevertheless, this lytic effect is lower than that shown by the enterocin S37 (74.2% using 10 μg/mL) [46], the lantibiotic nisin (6% using 3.35 μg/mL) (1 μM) [47] or P34 and P40 peptides from *Bacillus* (5.84 and 19.3% using 2.5 μg/mL, respectively) (corresponding to 1.7 and 3.12 μM) [17,48]. Bovicin HC5 represents an exception to this pattern (<4% at 200 μM) [49]. These *in vitro* experiments demonstrated that AS-48 exhibits a broad bactericidal activity in blood with no significant haemolytic effect against human erythrocytes,

suggesting high selectivity for bacteria *versus* human cells. It is known that the antimicrobial activity of cationic peptides can be attenuated by proteolytic enzymes and divalent cations (which can hinder the bioavailability of the bacteriocin by competing for anionic sites of interaction on membranes [50]. In addition, the presence of albumin, which has a high binding capacity for hydrophobic compounds, constitutes another check on bacteriocin efficacy in the bloodstream. For these reasons, one of the major challenges in developing AS-48 for use as a drug is to verify its stability and activity in whole blood. This peptide was investigated in assays that simulated conditions of high titre bacteraemia with different bacterial species. According to our results, AS-48 was stable in blood for the duration of the time assayed (24 h) and retained its antimicrobial activity. This behaviour is entirely different from that shown by the lantibiotic group of bacteriocins, which have reduced efficacy due to their propensity to bind blood components [50].

Our results indicate a lack of AS-48 cytotoxicity toward the primary epithelial human cell line MCF10A and the fibroblast line CCD18Co (currently widely used as controls for drug toxicity), confirming its inability to cause cell death or loss of viability in these human primary cell lines (>90% cell viability) at the highest AS-48 concentration tested (27 μM). This result indicates that AS-48 is considered non-cytotoxic according to standard international protocols (UNE-EN ISO 10993-5:2009). Thus, these data confirm that AS-48 is not toxic towards the non-tumour cell lines tested, in agreement with results described elsewhere, in which AS-48 was applied to normal human lung fibroblast MRC-5 cells [50], murine macrophage lines MHS and J774 [25], and the R1 cell line (ACC 56) derived from Chinook salmon liver [51]. Although some other peptides have shown a cell-line-specific cytotoxic effect, under the experimental conditions used here, AS-48 did not affect the normal cell lines tested at the concentrations assayed, even at 27 μM, a concentration much higher than its MICs towards bacteria. The MIC values of AS-48 were around 0.3 μM for *P. acnes*, one of the most susceptible bacteria [26], between 0.16 and 0.73 μM for *Staphylococcus* species [24] and between 4.47 and 8.90 μM for *M. tuberculosis* complex clinical and reference strains (the most resistant Gram-positive bacteria described) [25]. Moreover, potent synergies with lysozyme against *P. acnes*, with nisin against *Staphylococcus,* or with ethambutol against mycobacteria, have been measured, suggesting that further reductions in dosage are feasible. The resistance to AS-48 observed in eukaryotic cells was not unexpected, since eukaryotic membranes lack negatively charged lipids and contain cholesterol. Other studies have described the binding of positively charged bacteriocins by negatively charged glycosaminoglycan sulphate residues present in the proteoglycans of the eukaryotic glycocalyx, which protects eukaryotic cells against membrane damage [52]. However, in some eukaryotic organisms, such as parasites with negatively charged membranes (i.e. trypanosomatids), AS-48 is very effective (even more than against some bacteria) [53–55].

Likewise, in our *in vitro* inflammation disorders model, AS-48 did not significantly alter viability or induce nitrite accumulation in non-stimulated RAW macrophages, demonstrating an absence of pro-inflammatory effects. Interestingly, AS-48 decreased nitric oxide (NOx) production induced by LPS (up to 13.5 μM) (>96.51 μg/mL), which suggests an immunomodulatory function of AS-48. The observed AS-48 toxicity in *in vivo* assays performed using zebrafish embryos could be due to the widespread high rate of proliferation that occurs during organogenesis (which starts 1 day post-fertilization) and makes these embryos prone to structural malformations [37]. It is striking that even though assays using zebrafish embryos for toxicological purposes have been used for several years, there is a systematic lack of information on bacteriocins in such models (even for nisin). Furthermore, there are few references in the literature using this model with peptides. RhoB-crotamine, a defensin-like cationic peptide that displays cell-penetrating, antitumor, antimicrobial, antifungal, and antiparasitic properties at low micromolar range, was lethal to zebrafish embryos at all concentrations assayed (0.25–1.0 μM) [38]. In a recent safety study of the cyclic lipopeptide bacillomycin DC, the LC_{50} value after 96 h of exposure was 22.2 μg/mL [56]. In this case, the estimated lethal dose 50 (LD_{50}) value was between 3 and 6 μM, and the maximum tolerated dose (MTD) was 1.39 μM. Many factors may contribute to this behaviour, especially since other non-toxic proteins, such as bovine serum albumin, widely used as a carrier for *in vitro* experiments on cultured mammalian cells, strongly affects zebrafish embryos, even at the lowest concentrations tested (0.5%), resulting in a strong developmental delay [57]. Furthermore, the greater sensitivity of zebrafish embryos to AS-48 may be explained by the critical development stage of the embryos and the generally higher toxicity of cationic compounds during this proliferative phase compared with neutral compounds [37,38]. This may be especially true of immature cells, which are generally more sensitive to teratogenic effects than fully differentiated cells [37]. These results, together with the absence of data on the toxicity of cationic antimicrobial peptides in zebrafish eggs suggests that this model may not be the most appropriate to test this type of compound.

Since topical delivery may be one of the most plausible routes of administration of AS-48, to circumvent potential adverse systemic effects [1,58], its pro-inflammatory effects and ability to induce *in vivo* lymphocyte proliferation in mice, upon skin sensitization was assessed. The absence of lymphocyte proliferation in local lymph nodes after topical exposure to different concentrations of AS-48 indicates that this peptide does not induce skin sensitization or cause allergic contact dermatitis.

Finally, our *in vivo* test in mice to evaluate the impact of a cumulative AS-48 dose of 5 mg/kg (100 μg/mouse) intraperitoneally administered in 6 doses (one every 8 h), revealed that AS-48 does not produce toxic effects that cause weight loss or splenomegaly. Similar results have been obtained with rainbow trout after exposure to 13.98 μM of AS-48 for 96 h. Even in the treated fish, visible signs of abnormality/toxicity were not observed [51].

Conclusions

The positive results presented in this work highlight the potential of AS-48 as a feasible candidate for further pharmacological development (after carrying out the required clinical trials), to prevent and treat infections, even with multi-drug resistant microorganisms, particularly in skin and soft tissues [4]. Indeed, bacteriocins produced by LAB probiotic strains have been marketed for skin care, as topical formulations to prevent and treat skin diseases, including anti-ageing benefits [44]. Global interest in bacteriocins as natural inhibitory agents to fight diverse infections is changing perspectives within the pharmaceutical industry [1,44]. The susceptibilities of several highly sensitive clinical isolates of *S. aureus* [24] and *P. acnes* [26] to AS-48 have been successfully evaluated, and the application of AS-48 as a topical antibacterial agent for these types of skin infections has been patented [59]. The effect of AS-48 either alone or synergistically combined with other antimicrobial compounds such as lysozyme, nisin or ethambutol, could further reinforce its utility in diverse scenarios. AS-48 is also a potential new leishmanicidal agent, and its use has been suggested for the treatment of ulcers in non-disseminated cutaneous leishmaniasis [54]. Additional pharmacokinetic and *in vivo* efficacy studies will prove the real applicability of AS-48 to treat infections.

The results presented here represent pioneering work in the field of bacteriocin toxicity in *in vivo* models, as, despite extensive

use of bacteriocins in food and abundant literature suggesting their potential in medicine, there is limited research addressing this point.

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Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirments

Whole blood from human samples was obtained from the Biobank of Granada (Spain) (http://www.juntadeandalucia.es/salud/biobanco/) (Public Sanitary System of Andalusia) after obtaining the appropriate permits from the Ethical Research Committees of the Biobank and the University of Granada (Application number 32130034PV01).

Experimental procedures and protocols used in this study were performed according to the "Guide to the care and use of laboratory animals in research and teaching". The Commission of Ethics in Animal Experimentation of the University of Granada (Spain) approved the experimental protocol (Register number 14-CEEA-OH-2013).

Assays in vivo in BALB/c mice were performed according to rules and principles of the international guide for biomedical research in experimental animals, including use of the minimum number of animals necessary to produce statistically reproducible results, and the Three Rs concept (Replacement, Reduction, and Refinement).

Test for toxicity in zebrafish embryos follows the guidelines of the OECD Draft Guideline Fish Embryo Toxicity (FET) Test for the study of chemical substances (May 30, 2006).

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